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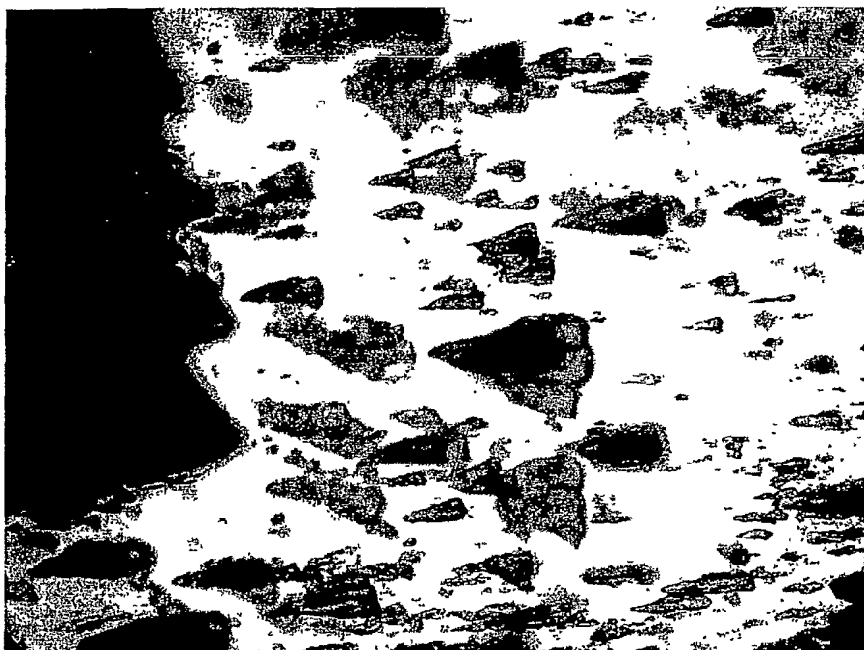
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[Continued on next page]

(54) Title: METHODS, COMPOSITIONS, AND KITS FOR PROTEIN CRYSTALLIZATION



(57) Abstract: The present invention provides methods, compositions, and kits for protein crystallization. The present invention involves electrophoretically focusing at least a first protein species within a matrix comprising at least 2 regions of different pH, the protein being present in amount sufficient to permit crystallization within said pH gradient.

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METHODS, COMPOSITIONS, AND KITS FOR PROTEIN
CRYSTALLIZATION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S.
5 provisional patent applications Serial No. 60/525,811
filed November 28, 2003, the disclosure of which is
incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] Knowledge of the three dimensional structure
10 of a protein is often necessary in order fully to
elucidate its biological function and its interaction
with other proteins, and with non-protein cofactors, in
a physiological pathway. Knowledge of a protein's 3D
structure is critical to the rational design of small
15 chemical entities capable of interacting with the
protein with high affinity, a process used both for
creation and for optimization of pharmaceutical
compounds intended for ultimate use in the clinic.

[0003] Although solution phase NMR spectroscopy has
20 shown promise in elucidating protein 3D structure, its
use is circumscribed by various technical limitations,
and X-ray crystallography remains the principal means

by which three dimensional protein structures are determined at the atomic level.

[0004] Yet crystallization of proteins remains an inexact and cumbersome art.

5 **[0005]** Typically, a protein desired to be crystallized is subjected to a wide variety of environmental conditions, including a wide range of solution chemistries, either serially or in a highly parallel series of assays. The large number of assays
10 demands a commensurately large quantity of protein. Positive results occur episodically, and often only after weeks of incubation. Even when a positive result is obtained in the form of a crystal, preparing and mounting the crystal on an x-ray machine can be an
15 arduous, time-consuming process that often results in crystal degradation or even loss.

[0006] Thus, there exists a need in the art for a simple, rapid, versatile method of producing protein crystals that neither requires multiple assays, a
20 large amount of protein, nor difficult crystal manipulation methods.

SUMMARY OF THE INVENTION

[0007] The present invention solves these and other needs in the art by providing, in a first aspect, a
25 method of crystallizing one or more protein species of interest.

[0008] The method comprises electrophoretic focusing of at least a first protein species within a pH gradient, such as a continuous pH gradient, or within a
30 polymer matrix that includes at least 2 regions of different pH, the protein being present in an amount

sufficient to permit crystallization within the gradient.

[0009] Without intending to be bound by theory, it would appear that as a protein approaches its
5 isoelectric point and thereby becomes more concentrated, the loss of net charge reduces its aqueous solubility, facilitating crystallization. Thus, applied to the gradient in sufficient amount, a protein may be observed to crystallize at or near its
10 isoelectric focal point, particularly when solubilizing and denaturing agents that are typically used in analytical isoelectric focusing (IEF) are omitted during the electrophoretic procedure.

[0010] The method does not require that
15 crystallization be effected exactly at the isoelectric point, however. Rather, by exposing the protein to a continuous pH gradient, the method submits the protein in a single procedure to a continuously varying spectrum of environmental conditions as the protein
20 migrates through the pH gradient, at least one of which conditions proves effective at instigating crystallization. The protein crystals created within the pH gradient or the polymer matrix that includes at least 2 regions of different pH, can, in some
25 embodiments, thereafter be used to nucleate further crystallization of the respective protein from solution. In other words, these protein crystals can be used as seed crystals. Therefore, in certain aspects, provided herein is a method of forming seed
30 crystals of one or more protein species of interest.

[0011] In a related embodiment, the method comprises electrophoretically transporting at least a first protein species within a pH gradient, such as a

continuous pH gradient, or within a polymer matrix that includes at least 2 regions of different pH, wherein the isoelectric point of the protein falls outside of the pH gradient or the pH regions of the polymer matrix, the protein being present in an amount sufficient to permit formation of protein crystals at a terminal region of the pH gradient or polymer matrix.

5 [0012] The method can further include removing the terminal region of the polymer matrix that includes the protein crystals, and analyzing the structure of the proteins in the protein crystals, for example using x-ray diffraction. The method can include using a protein crystal transport tool to transport the protein crystals embedded in the polymer matrix, to an x-ray
10 diffraction holder.

[0013] The method is simple, rapid, applicable to a wide variety of proteins, including proteins in an inhomogeneous admixture, and requires minimal user intervention, thus providing significant advantages
15 over methods currently used in the art.

[0014] In one series of embodiments, the focusing is conveniently performed in a matrix having an immobilized continuous pH gradient, such as in immobilized pH gradient (IPG) strip.

25 [0015] In another aspect, the invention provides protein crystal compositions created using the methods of the present invention.

[0016] In one series of embodiments, the composition comprises at least one crystal of at least a first
30 protein species, and a polymer matrix comprising a plurality of species of pH-conferring moieties, each of the plurality having a distinct pKa. In certain embodiments, the plurality of covalently linked, pH-

conferring moieties collectively establish a continuous pH gradient along at least one dimension of the polymeric matrix.

5 **[0017]** In certain particularly useful embodiments, at least one crystal of the at least first protein species is of sufficient size as to permit X-ray diffraction analysis, and the protein is crystallized in a native, non-denatured, conformation.

10 **[0018]** The polymer matrix can be an acrylamide polymer, such as an acrylamide polymer comprising a plurality of covalently linked pH-conferring moieties, such as the acrylamide matrix of an IPG strip. At least one of the pH-conferring moieties can, for example, be an acrylamido buffer monomer that is co-
15 polymerized with the acrylamide monomers.

20 **[0019]** The composition may, in various embodiments, lack detergent in amounts and of composition sufficient to denature the first protein species, thus preserving a native conformation of the crystallized protein. Analogously, the composition may, in various additional
embodiments, lack chaotropic agents in amounts and of composition sufficient to denature the first protein species, thus preserving a native conformation of the crystallized protein.

25 **[0020]** In other words, in such compositions the amounts of detergent or chaotropic agents present, if any, are insufficient to effect denaturation or to stabilize other non-native conformations of the first protein species therein.

30 **[0021]** In yet a further aspect, the invention provides kits for facilitating the practice of the methods of the present invention.

[0022] In one series of embodiments, the kit comprises a polymer matrix that includes at least 2 regions of different pH, such as a polymer matrix having a continuous pH gradient and one or more of instructions for performing the methods of the present invention, a protein crystal transport tool for transporting a crystal embedded gel to an x-ray diffraction holder, and an x-ray crystallography holder.

10 **[0023]** The kit can further include an aqueous solvating composition, the solvating composition being adapted to and permissive for subsequent crystallization, according to the method of the present invention, of a protein species dissolved therein.

15 **[0024]** For example, the solvating composition may, in typical embodiments, lack detergent in amounts and of composition sufficient to denature a protein. The solvating composition may typically lack chaotropic agents in amounts and of composition sufficient to denature a protein. The solvating composition will typically be of low ionic strength, and only slightly buffered.

[0025] In other words, in such solvating compositions the amounts of detergent or chaotropic agents present, if any, are insufficient to effect denaturation or to stabilize other non-native conformations of the first protein species therein.

[0026] To facilitate the crystallization of proteins that may initially be present in compositions that are insufficiently permissive of crystallization according to the methods of the present invention, certain
30 embodiments of the kits of the present invention may

include means for exchanging a protein into said aqueous solvating composition.

[0027] In a variety of embodiments, the polymer matrix is part of an IPG strip, and the kit comprises one or more IPG strips. In typical embodiments, the kit comprises a selection of IPG strips having different continuous pH ranges, including both linear and nonlinear pH ranges, so as to facilitate the crystallization of a wide variety of proteins of disparate pI.

[0028] Usefully, the kit comprises means for effecting isoelectric focusing in IPG strips.

BRIEF DESCRIPTION OF THE DRAWINGS

[0029] The above and other objects and advantages of the present invention will be apparent upon consideration of the following detailed description taken in conjunction with the accompanying drawings, in which like characters refer to like parts throughout, and in which:

[0030] FIG. 1 is a photomicrograph showing crystals of soybean trypsin inhibitor formed within a pH 4.5 - 5.5 IPG strip (ZOOM[®] Strip, Invitrogen Corp., Carlsbad, CA) after fewer than 4 hours of isoelectric focusing in an Invitrogen Corporation ZOOM[®] IPGRunner cassette, according to one embodiment of the methods of the present invention;

[0031] FIG. 2A is a photomicrograph showing seed crystals of lysozyme formed within a pH 9 - 12 IPG strip after fewer than 3 hours isoelectric focusing in a ZOOM[®] IPGRunner cassette (Invitrogen Corp., Carlsbad, CA) and FIG. 2B is a photomicrograph of crystals of

lysozyme grown from these initial seeds, according to one embodiment of the present invention;

[0032] FIG. 3 is a photomicrograph showing a capillary-mounted crystal of cytochrome P450 3A4. This crystal was produced within a pH 3 - 10 non-linear IPG strip (ZOOM[®] Strip, Invitrogen Corp., Carlsbad, CA) after 1 hour of isoelectric focusing in a ZOOM[®] IPGRunner cassette (Invitrogen Corp., Carlsbad, CA), according to one embodiment of the present invention; and

[0033] FIG. 4 is a photomicrograph showing crystals of Hemoglobin S formed under the electrode at the acidic end of a pH 9-12 IPG Strip after 4 hours of isoelectric focusing in a ZOOM[®] IPGRunner cassette (Invitrogen Corp., Carlsbad, CA), according to one embodiment of the present invention.

DETAILED DESCRIPTION

[0034] In a first aspect, the invention provides a method for crystallizing one or more protein species of interest.

[0035] The method comprises electrophoretic focusing of at least a first protein species within a pH gradient, the protein being present in sufficient amount to permit crystallization within the gradient. The protein crystals created within the pH gradient can, in some embodiments, thereafter be used to nucleate further crystallization of the respective protein from solution. Thus, the protein crystals can be used as seed crystals.

[0036] Accordingly, in certain aspects of the present invention, provided herein is a method of forming seed crystals of one or more protein species of

interest by electrophoretic focusing of at least a first protein species within a pH gradient, the protein being present in sufficient amount to permit crystallization within the gradient, and contacting the protein crystals with additional protein of interest at a concentration sufficient to instigate additional crystal formation from the protein crystals.

5 [0037] Typically, the protein crystals in the pH gradient are transferred to a new vessel in which a solution of the protein of interest is added to form additional crystals using the protein crystals formed in the pH gradient as seed crystals. The protein crystals in the pH gradient are typically isolated before they are transferred to the new vessel for additional crystal formation.

10 [0038] In certain aspects, at least one protein of interest is electrophoretically transported within a polymer matrix that includes at least 2 regions of different pH. In certain aspects, the polymer matrix includes a pH gradient. The pH gradient can be linear or nonlinear, and will typically, although not necessarily, include the isoelectric point of the protein or proteins desired to be crystallized. The pH gradient may be comprised of smooth transitions of pH or pH steps, and the number of steps can be as few as one. For instances in which the isoelectric point of a protein lies outside the gradient's pH range, protein crystals can still form within the polymer matrix in close proximity to one of the electrodes, which is typically a terminal portion of the polymer matrix (e.g., gel), as disclosed herein.

15 [0039] In a related embodiment, the method comprises electrophoretically transporting at least a first

protein species within a pH gradient, such as a continuous pH gradient, or within a polymer matrix that includes at least 2 regions of different pH, such as a polymer matrix that includes a continuous or

5 discontinuous pH gradient, wherein the isoelectric point of the protein falls outside of the pH gradient or the pH regions of the polymer matrix, the protein being present in an amount sufficient to permit formation of protein crystals at a terminal region of

10 the polymer matrix, at an electrode that contacts an end of the polymer matrix, and/or at an object that is located between an end of the polymer matrix and an electrode, such as a wick located between an electrode and a gel end. Such a wick is present, for example,

15 when using the ZOOM® IPG Runner (Invitrogen Corp., Carlsbad, CA). The method can further include removing the terminal region of the polymer matrix that includes the protein crystals and analyzing the structure of the protein in the protein crystals, for example, using x-

20 ray diffraction. The isoelectric point of the protein falls outside of the pH gradient or the pH regions of the polymer matrix when the isoelectric point of the protein is lower or higher than the lowest and highest pH regions, respectively, of the polymer matrix.

25 **[0040]** In certain aspects, the method of crystallizing at least one protein provided herein can include using a protein crystal transport tool to excise polymer matrix sections containing protein crystals and to transport the protein crystals embedded

30 in the polymer matrix to an x-ray diffraction holder. Furthermore, the protein crystals can be analyzed using x-ray diffraction or solubilized and analyzed by solution phase NMR.

[0041] The gradient can have a pH range of as little as 0.5 pH unit, 1.0 pH unit, 1.5 pH unit, and may have a range of as much as 2 pH units, 3 pH units, 4 pH units, 5 pH units, 6 pH units, even as much as 7, 8, or 9 pH units, with intermediate non-integral ranges permissible.

[0042] For example, a wide pH gradient may prove particularly useful in embodiments in which the pI of the protein species desired to be crystallized is unknown, and in embodiments, further described below, in which the protein species desired to be crystallized is present in inhomogeneous admixture with other protein species with divergent pI values. Conversely, a shallow pH gradient may prove particularly useful in embodiments in which the pI of the protein species desired to be crystallized is precisely known, and in embodiments in which the protein species desired to be crystallized is present in substantially purified form.

[0043] The pH gradient can extend as low as pH 1, pH 1.5, even as low as pH 2, although more typically the pH gradient will extend only as low as pH 2.5 or 3, and can extend as high as pH 13, more typically as high as pH 12, and more typically no more than about pH 10, with intermediate and non-integral values permissible.

[0044] The isoelectric point of the protein desired to be crystallized may fall in the middle of the pH gradient or towards its acidic or basic end.

[0045] Preferably, the isoelectric focusing is performed in a matrix having an immobilized pH gradient.

[0046] The matrix can be a polymer matrix.

[0047] For example, the matrix can be a polyacrylamide matrix produced by co-polymerization of

monoolefinic acrylamide monomers with diolefinic or polyolefinic acrylamide monomers.

[0048] Monoolefinic monomers useful in the preparation of polyacrylamide matrices for use in the methods of the present invention include acrylamide, methacrylamide and derivatives thereof such as alkyl derivatives or hydroxyalkyl derivatives, e.g. N,N-dimethylacrylamide, N-hydroxypropylacrylamide, and N-hydroxymethylacrylamide. The monoolefinic monomer can also be selected from acrylic and methacrylic acid derivatives, e.g., alkyl esters such as ethyl acrylate and hydroxyalkyl esters such as 2-hydroxyethyl methacrylate.

[0049] Diolefinic or polyolefinic cross-linking monomers useful in the preparation of polyacrylamide matrices for use in the methods of the present invention include compounds containing two or more acryl or methacryl groups, such as methylenebisacrylamide, N,N'-diallyltartardiamide, N,N'-1,2-dihydroxyethylene-bisacrylamide, N,N-bisacrylyl cystamine, trisacryloyl-hexahydrotriazine.

[0050] In other embodiments, the polymer matrix is prepared by co-polymerization of a monoolefinic acrylamide monomer, such as those described above, with a polysaccharide substituted to contain vinyl groups, for example allyl glycidyl dextran, as described in U.S. Patent Nos. 4,504,641 and 4,542,200, the disclosures of which are incorporated herein by reference in their entireties. Other containing matrices useful in the methods of the present invention include the covalently cross-linked, mixed-bed agarose polyacrylamide matrices described in U.S. Pat. No.

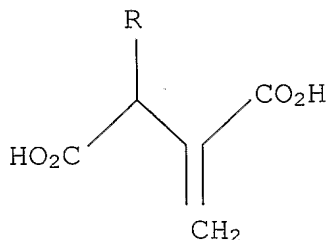
5,785,832, the disclosure of which is incorporated herein by reference in its entirety.

[0051] In various embodiments, the continuous pH gradient is created by the covalent linkage within the polymer matrix of a plurality of species of buffering (synonymously, "pH-conferring") moieties, the various species having disparate pKa values.

[0052] In one series of embodiments, the co-polymerized pH-conferring monomer is an acrylamido buffer monomer. As is known in the art, acrylamido buffers are non-amphoteric weak acids and bases having a vinyl moiety for covalent incorporation into the gel matrix.

[0053] Acrylamido buffer monomers useful in the polymer matrices for use in the methods of the present invention are known in the art. A number of commercially available acrylamido buffers (Amersham Biosciences, Piscataway, NJ, USA, and Sigma-Aldrich, St. Louis, MO, USA) include: 2-acrylamido-2-methylpropane sulfonic acid; 2-acrylamidoglycolic acid; N-acryloylglycine; 4-acrylamidobutyric acid; 2-morpholinoethylacrylamide; 3-morpholinopropylacrylamide; N,N-dimethylaminoethylacrylamide; N,N-dimethylaminoethylpropylacrylamide; and N,N-diethylaminopropylacrylamide.

[0054] In other embodiments, the co-polymerized pH-conferring monomer is a dicarboxylic acid, usefully with the formula



5 in which R is selected from the group consisting of:
-H, -OH, -CH₂OH, -CO₂H, -NHR', -OCH₃, and -NR'R'', -Cl, -
F, -I, and wherein R' and R'' are each independently
selected from the group consisting of -CH₃, -CH₂CH₃,
-CH₂OH, -CH₂CH₂OH, -CH₂CH₂CH₃.

10 **[0055]** In certain embodiments, the polymer matrix is
adherent to at least one solid backing member.
Adherence can be by covalent linkage to a single
backing member, which can usefully be made of a
flexible plastic having pendent vinyl groups.

15 **[0056]** In a subset of such embodiments, the plastic
backing member is fashioned in the shape of a strip
that is greater in length along the axis of the pH
gradient than in the dimensions orthogonal thereto.

[0057] The strip can, for example, have approximate
20 lengths along the axis of the pH gradient of, for
example, 10 mm, 20 mm, 30 mm, 40 mm, 50 mm, 60 mm, 70
mm, 80 mm, 90 mm, 100 mm, 110 mm, 120 mm, 130 mm, 140
mm, 150 mm, 160 mm, 180 mm, 200 mm, 220 mm, 240 mm, or
even longer, with intermediate lengths permissible.

25 **[0058]** Longer strips can present advantages in
resolving, and crystallizing, a protein species present
in inhomogeneous admixture with a large number of other
proteins. For example, a pH gradient embodied on a
longer strip may provide isoelectric focusing at a
30 higher resolution. Such a higher resolution may be
particularly useful when formation of protein crystals
is particularly sensitive to the pH environment.

Shorter strips can present advantages in speed of focusing and crystallization.

[0059] In some embodiments, the strips can have approximate widths of, for example, 1.0 mm, 1.5 mm, 2 mm, 2.5 mm, 3 mm, 3.5 mm, 4 mm, 4.5 mm, 5 mm, or even wider and approximate thicknesses of 0.1 mm, 0.15 mm, 0.2 mm, 0.25 mm, 0.3 mm, 0.35 mm, 0.4 mm, 0.45 mm, 0.5 mm, 0.55 mm, 0.6 mm, 0.65 mm, 0.7 mm, 0.75 mm, 0.8 mm or even thicker, with intermediate lengths permissible.

10 **[0060]** More narrow strips can present advantages in embodiments in which protein is limited in amount.

[0061] In typical embodiments, further described below, IPG strips useful in the methods of the present invention can be provided in dehydrated form, to be rehydrated prior to electrophoretic focusing of proteins therein. Neither complete removal of moisture, during dehydration, nor complete saturation with liquid, during rehydration, is required or intended.

20 **[0062]** Such immobilized pH gradient (IPG) strips can be cast using methods well known in the art. IPG strips useful in the methods of the present invention are also available commercially in a variety of lengths and pH ranges, typically in such dehydrated form.

25 **[0063]** For example, ZOOM[®] Strips from Invitrogen Corporation (Carlsbad, CA) are 7 cm long and are available in a pH 3 - 10 nonlinear range, a pH 4 - 7 linear range, a pH 6 - 10 linear range, a pH 4.5 - 5.5 linear range, a pH 5.3 - 6.3 linear range, and a pH 6.1 - 7.1 linear range.

30 **[0064]** ReadyStrip IPG Strips from Bio-Rad (Hercules, CA) are available in lengths of 7 cm, 17 cm, and 18 cm, in pH ranges of pH 3 - 10, pH 6.3 - 8.3, pH 3 - 6,

pH 3.9 - 5.1, pH 4 - 7, pH 4.7 - 5.9, pH 5 - 8, pH 5.5 - 6.7, pH 7 - 10, and in a nonlinear range of pH 3 - 10.

[0065] Additionally, Amersham Biosciences
5 (Piscataway, NJ) sells IPG strips in 7, 11, 13 and 18 cm lengths with pH ranges of pH 4-7, pH 3 - 10 linear, pH 3 - 10 nonlinear, and pH 6 - 11.

[0066] In presently preferred embodiments, the IPG
10 strips are formulated to permit rehydration at room temperature in no more than about 8 hours, preferably no more than about 7 hours, 6 hours, 5 hours, 4 hours, even more preferably no more than about 3, 2, or even 1 hour.

[0067] Such rapidly rehydratable IPG strips are
15 typically cast from a basic solution and an acidic solution, the basic solution comprising at least three acrylamido buffers with a combined concentration of at least about 32 mM, at least about 35 mM, even at least about 40 mM or more, as further described in commonly
20 owned U.S. provisional patent application nos. 60/509,512, filed October 7, 2003, 60/510,674, filed October 9, 2003, and commonly-owned and co-pending U.S. patent application no 10/961,308, filed October 7, 2004, the disclosures of which are incorporated herein
25 by reference in their entireties.

[0068] Although IPG strips, including commercially
available IPG strips, provide convenience in the practice of the methods of the present invention, they are not required. The methods of the present invention
30 can be practiced in slab gels, with or without one or more solid support backings, in tubes that can readily be opened, in capillaries, and in any other geometry as

may permit isoelectric focusing in a continuous pH gradient.

[0069] Because isoelectric focusing in a pH gradient separates proteins based upon differences in their isoelectric points, the protein species desired to be crystallized using the methods of the present invention need not be pure, and may indeed be present in inhomogeneous admixture with at least one additional protein species having a different isoelectric point, a major advantage of the methods of the present invention over methods currently used in the art. In some embodiments, it may also be desirable to crystallize one or more of the additional protein species; in some of these embodiments, each of the protein species can be concurrently crystallized within the continuous pH gradient at its respective isoelectric point.

[0070] In some embodiments of the present invention, the non-denaturing conditions may allow protein complexes to preserve their quaternary structure, and thus the components of the complex may not separate under the conditions used in the present invention. Under such conditions, isoelectric focusing of a protein complex using the methods of the present invention may permit crystallization of the protein complex at a particular pH value in the pH gradient.

[0071] The proteins desired to be crystallized may, therefore, be initially present within a cell lysate.

[0072] The first and optional additional protein species may, for example, be proteins naturally expressed within a cell and be present within a lysate thereof.

[0073] Alternatively, the first and optional additional protein species desired to be crystallized

may be recombinantly expressed proteins. In such embodiments, the recombinant expression can be effected within a cell, such as a cell selected from the group consisting of: animal cell, including mammalian cell, such as CHO cell; fungal cell, such as a *Saccharomyces cerevisiae* cell or *Pichia* cell; an insect cell; a plant cell; and a prokaryotic cell, such as a eubacterial cell, such as an *E. coli* cell.

[0074] In yet another alternative, the first and optional additional protein species desired to be crystallized may be recombinantly expressed within one or more cell-free lysates, such as a coupled transcription-translation system, such as the commercially available ExpressWay coupled transcription-translation system (Invitrogen Corp., Carlsbad, CA).

[0075] IPG sample preparation solutions used in the art for analysis of proteins typically include one or more denaturing agents, such as urea or thiourea, one or more nonionic or zwitterionic detergents, such as CHAPS, NP-40, CHAPSO, and detergent sulfobetaines such as SB3-10, one or more reducing agents, such as dithiothreitol (DTT) or dithioerythritol (DTE), and carrier ampholytes.

[0076] In embodiments of the methods of the present invention in which proteins are desired to be crystallized in their native state -- for example, in order subsequently to determine their native 3D structure -- denaturing agents and reducing agents are usefully omitted, both from the protein sample applied to the continuous pH gradient and, in embodiments in which the pH gradient is present within an IPG strip,

usefully omitted from the strip rehydration solution as well.

[0077] Analogously, in embodiments of the methods of the present invention in which proteins are desired to be crystallized in their native state, carrier ampholytes, which tend to adhere to proteins, can usefully be omitted from the protein sample applied to the continuous pH gradient and, in embodiments in which the pH gradient is present within an IPG strip, usefully omitted from the strip rehydration solution as well.

[0078] And in embodiments in which proteins do not readily crystallize within the continuous pH gradient, solubilizing agents such as detergents can usefully be omitted from the protein sample applied to the continuous pH gradient and, in embodiments in which the pH gradient is present within an IPG strip, usefully omitted from the strip rehydration solution as well.

[0079] If proteins desired to be crystallized according to the methods of the present invention are present in a composition having one or more undesired agents, such as salts or detergents, the protein can be exchanged into a desired solution composition prior to its application to the continuous pH gradient using art-accepted techniques such as dialysis or, as described in Example 3, below, centrifugation using a semi-permeable membrane with known molecular weight cutoff.

[0080] Thus, in some embodiments of the methods of the present invention, the first and optional additional protein species desired to be crystallized are electrophoretically focused in the substantial absence of detergent. In some embodiments, the first

and optional additional protein species are electrophoretically focused in the substantial absence of chaotropes.

5 **[0081]** In some embodiments, the protein can even be dissolved in, and thus focused in, deionized water, as shown in Example 1, below.

[0082] Conversely, agents desired to be present can be added before the application of the proteins to the continuous pH gradient.

10 **[0083]** For example, non-detergent zwitterions, such as non-detergent sulfobetaines, can be added. Such non-detergent sulfobetaines include, for example, dimethylethylammonium propane sulfonate; 3-(1-pyridino)-1-propane sulfonate; dimethylbenzylammonium
15 propane sulfonate; 3-(4-*tert*-butyl-1-pyridino)-1-propane sulfonate; and 3-(1-methylpiperidinium)-1-propane sulfonate.

[0084] In other embodiments, volume-excluding or macromolecular crowding agents that further concentrate
20 the proteins can be added, additionally or in the alternative. For example, polyethylene glycols or other polysaccharides can be added.

[0085] In embodiments of the methods of the present invention in which the continuous pH gradient is
25 immobilized within an IPG strip, electrophoretic focusing of the proteins can usefully be performed by hydratingly lodging the prior-cast, dehydrated, IPG strip within an enclosing member that permits spaced electrical communication with the strip, and then using
30 the spaced electrical communication to establish a voltage gradient in the gel matrix of the strip that is sufficient to effect electrophoretic focusing of proteins therein.

[0086] In these embodiments, the method will typically further comprise inserting the IPG strip in its dehydrated state within the enclosing member, and then contacting the enclosed dehydrated IPG strip with an aqueous solution for a time sufficient to lodge the strip within the enclosing member. The aqueous solution used to rehydrate the strip can usefully comprise the first and optional additional protein species to be crystallized.

10 **[0087]** Such embodiments of the methods of the present invention are readily practiced using the IPGRunner system commercially available from Invitrogen Corp. (Carlsbad, CA), which provides means for enclosing a plurality of IPG strips, the enclosing means permitting spaced electrical communication separately with each of the enclosed strips through respective first and second entries. The system further provides means responsive to an external compressive force for effecting spaced electrical communication by a single anode and single cathode simultaneously with each of the enclosed strips, wherein the electrical communication means is capable of distributing an external compressive force to urge the anode and the cathode toward the enclosing means with greater pressure at the first and second entries than elsewhere on the enclosing means.

20 **[0088]** Advantageously, the IPGRunner system obviates the use of oil, contact to which may interfere with crystallization of subsequent isolation of crystals formed within the strips. Additionally, the IPG strips, with crystals formed therein, can be frozen within the cassette.

[0089] Variants of such system prove useful in other embodiments of the present invention. For example, in certain variants, the enclosing member encloses a single IPG strip, rather than a plurality of such strips.

[0090] Particularly using the IPGRunner system and its variants (although not limited thereto), the methods of the present invention allow the first and optional additional protein species to be crystallized in fewer than 10 hours, 9 hours, 8 hours, 7 hours, 6 hours, 5 hours, even as few as 4 hours, 3 hours, and even as few as 2 or 1.5 hours, as demonstrated in Examples 1 - 3 herein below, with intermediate and non-integral values achievable. The rapidity and simplicity with which the protein species desired to be crystallized is exposed to a continuous gradient of environmental conditions, and thus crystallized, presents significant advantages over methods currently used in the art.

[0091] The methods of the present invention can be practiced at room temperature or, depending upon the protein desired to be crystallized, at other temperatures. For example, the proteins can be electrophoretically focused at 4°C, 10°C, 15°C, even 20°C, 25°C, even 30°C or more, with intermediate and non-integral values permissible and with colder temperatures facilitating crystallization of certain proteins.

[0092] Although the methods of the present invention permit a desired protein species to be purified and crystallized in a single step from an inhomogeneous mixture, in some embodiments the method may further comprise an antecedent purification step, typically one

in which the first and optional additional protein species desired to be crystallized are partially purified from an inhomogeneous protein mixture.

5 [0093] Prior purification can be effected by any means known in the art.

[0094] For example, the protein species desired to be crystallized may be partially purified before isoelectric focusing by one or more well known chromatographic or electrophoretic methods, including
10 gel filtration, ion exchange, reverse phase HPLC, or capillary or capillary zone electrophoresis. The protein species desired to be crystallized may in addition or in the alternative be size fractionated by dialysis or by centrifugation through columns having
15 semi-permeable membranes.

[0095] Recombinant proteins desired to be crystallized according to the methods of the present invention may additionally or in the alternative be partially or completely purified before isoelectric
20 focusing using fused affinity tags, such as the 6xHis tag, myc tag, FLAG tag, or tetracysteine tag (CCXXCC, where "X" represents any amino acid except cysteine). Tetracysteine-tagged fusion proteins can be purified using biarsenical affinity supports, such as the FLAsH[™]
25 (LUMIO[™] green) affinity resin described in *Protein Sci.* 9:213-217 (2002), and further described in commonly-owned and co-pending U.S. application no. 10/971,606, filed October 22, 2004, the disclosures of which are incorporated herein by reference in their entireties.
30 In such embodiments, the biarsenical compound does not need to be a biarsenical derivative of a fluorophore; it can be any biarsenical with spacing suitable for binding to a tetracysteine motif.

[0096] In certain pre-purification embodiments of the methods of the present invention, the protein species desired to be crystallized is first partially purified by solution phase isoelectric focusing
5 fractionation. See Zuo et al., *Anal. Biochem.* 284: 266-278 (2000); Zuo et. al., *Electrophoresis* 22: 1603-1615 (2001); Zuo et al., *Proteomics*: 2: 58-68 (2002); Zuo et al., *Journal of Chromatography B* 782: 253-265 (2002); Ali-Khan et al., Current Protocols in Protein
10 Science 22.1: 1-19 (2002), the disclosures of which are incorporated herein by reference in their entireties. Devices and kits for fluid phase isoelectric focusing are available commercially (ZOOM® IEF Fractionator, Invitrogen Corp., Carlsbad, CA).

15 [0097] Proteins crystallized according to the methods of the present invention, whether crystallized directly or only after prior partial purification, may thereafter be isolated from the continuous pH gradient.

[0098] In embodiments in which the pH gradient is
20 fixed within a polymer matrix, as in the polyacrylamide gel of an IPG strip, the portion of the polymer matrix comprising the protein crystals can be mechanically excised. This mechanical excision can be performed, for example, using one or more protein crystal
25 transport tools disclosed herein. In embodiments in which a plurality of protein species are concurrently crystallized within a single polymer matrix, each such crystallized protein species can be separately excised there from. In some embodiments, the excised protein
30 crystals can thereafter be washed with a solvent that supports maintenance of the crystal structure.

[0099] The protein crystals created within the continuous pH gradient can, in some embodiments,

thereafter be used to nucleate further crystallization of the respective protein from solution, as demonstrated in Example 2, below.

[0100] The crystals, whether obtained directly from
5 the continuous pH gradient or from a solution seeded with crystals so obtained, are in many embodiments of the methods of the present invention thereafter submitted to analysis, typically, but not exclusively, analysis to determine the 3D structure of the
10 crystallized protein.

[0101] Analysis may, for example, comprise diffraction analysis, such as X-ray diffraction analysis, including, e.g., X-ray diffraction analyses using third-generation synchrotron sources, undulator
15 beam lines, multi-wavelength anomalous dispersion (MAD) data collection, and CCD detection.

[0102] In some embodiments, the crystals will be cryo-protected, for example by flash cooling and storage at cryogenic temperatures before analysis. For
20 example, the crystal can be mounted into a cryo-loop, flash cooled at liquid nitrogen temperature, and subsequently stored in liquid nitrogen. In certain aspects in which the protein crystals are formed in a polymer matrix having an immobilized pH gradient, such
25 as an isoelectric focusing gel or an IPG strip, one or more pieces of the polymer matrix that include the protein crystals are dried and directly analyzed by X-ray crystallography. For example, the polymer matrix pieces can be placed onto a cryo-loop for direct x-ray
30 crystallographic analysis. The polymer matrix with the protein crystals can be dried/stored at room temperature until direct x-ray crystallographic analysis.

[0103] In some embodiments, the first (and optional additional protein species) desired to be crystallized is crystallized as part of a complex, such as a complex with a compound chosen from a chemical library; in such
5 embodiments, the protein species desired to be crystallized are first complexed, and then subjected to isoelectric focusing in a continuous pH gradient.

[0104] Analysis may, in addition or in the alternative, include nuclear magnetic resonance
10 spectroscopy and/or scanning electron microscopy.

[0105] In another aspect, the invention provides protein crystal compositions created using the methods of the present invention.

[0106] In a first series of embodiments, the
15 composition comprises at least one crystal of at least a first protein species, and a polymer matrix comprising a plurality of species of pH-conferring moieties, each of the plurality having a distinct pKa. The at least one protein crystal is typically embedded
20 within the polymer matrix.

[0107] In certain embodiments, at least one crystal of the at least first protein species is of sufficient size as to be light microscopically observable. In particularly useful embodiments, at least one crystal
25 of the at least first protein species is of sufficient size as to permit X-ray diffraction analysis.

[0108] The protein crystal is usefully a crystal of the protein in a native, non-denatured, conformation. The native, non-denatured, conformation can, for
30 example, be a conformation of the protein alone. In other embodiments, the native, non-denatured, conformation can be the conformation of the protein complexed with a non-protein ligand, such as a natural

cofactor or a mimetic thereof. In yet other embodiments, the native, non-denatured, conformation can be the conformation of the protein complexed with another protein species.

5 **[0109]** As described above with respect to the methods of the present invention, the polymer matrix can be an acrylamide polymer, such as an acrylamide polymer comprising a plurality of covalently linked pH-conferring moieties, each of the plurality of species
10 having a distinct pKa values.

[0110] In certain embodiments, the plurality of covalently linked pH-conferring moieties collectively establish a continuous pH gradient along at least one dimension of the polymeric matrix.

15 **[0111]** At least one of the pH-conferring moieties can, for example, be an acrylamido buffer monomer that is co-polymerized with the acrylamide monomers. In some embodiments, each of the plurality of pH-conferring moieties is an acrylamido monomer.

20 **[0112]** The acrylamido monomers can be selected, for example, from the group consisting of 2-acrylamido-2-methylpropane sulfonic acid; 2-acrylamidoglycolic acid; N-acryloylglycine; 4-acrylamidobutyric acid; 2-morpholinoethylacrylamide; 3-morpholino-
25 propylacrylamide; N,N-dimethylaminoethylacrylamide; N,N-dimethylaminoethylpropylacrylamide; and N,N-diethylaminopropylacrylamide.

[0113] The polymer matrix can be adherent to at least one support backing, usefully covalently attached
30 to a single support backing. In certain IPG strip embodiments, the support backing is a flexible plastic fashioned as a strip having greater length in the axis

of the pH gradient than in directions orthogonal thereto.

[0114] The pH gradient within the polymer matrix can have a pH range of up to 12 pH units, although typically the pH range, as in commercial IPG strips, will be no more than about 7, 8, 9, or 10 pH units. Conversely, the gradient can have a pH range of as little as 0.5 pH units, although ranges of at least 1 pH unit will be more typical.

[0115] The composition will, in various embodiments, lack detergent in amounts and of composition sufficient to denature the first protein species, thus preserving a native conformation of the crystallized protein. Although these embodiments comprehend compositions completely lacking in detergent, other such embodiments include one or more detergents in amounts and of composition that do not effectuate denaturation of the first protein species.

[0116] Analogously, the composition will, in various embodiments, lack chaotropic agents in amounts and of composition sufficient to denature the first protein species, thus preserving a native conformation of the crystallized protein. Although these embodiments comprehend compositions completely lacking in chaotropic agents, other such embodiments include one or more chaotropes in amounts and of composition that do not effectuate denaturation of the first protein species.

[0117] The compositions may further comprise a non-detergent zwitterion, such as a non-detergent sulfobetaine. Such nondetergent sulfobetaines include, for example, dimethylethylammonium propane sulfonate; 3-(1-pyridino)-1-propane sulfonate;

dimethylbenzylammonium propane sulfonate; 3-(4-*tert*-butyl-1-pyridino)-1-propane sulfonate; and 3-(1-methylpiperidinium)-1-propane sulfonate.

5 **[0118]** In other embodiments, the compositions comprise volume-excluding or crowding agents such as polyethylene glycols, monosaccharides, or other polysaccharides.

10 **[0119]** In yet further embodiments, the composition may further comprise at least one crystal of at least second protein species, spatially segregated from at least one crystal of the first protein species. As in the compositions comprising one or more crystals of a single protein species, in some embodiments at least one of the crystals of the at least second protein
15 species is of a size sufficient to be observed by light microscope; and in some embodiments, at least one of the crystals of the at least second protein species is of sufficient size to permit X-ray diffraction analysis.

20 **[0120]** In some embodiments, the compositions of the present invention are frozen.

25 **[0121]** In yet a further aspect, the invention provides methods for selling polymer matrices and tools for practicing the methods provided herein. In a first series of embodiments, the method includes providing a means, typically an Internet based means, for linking the ordering of a polymer matrix having at least 2 regions of different pH, such as a continuous pH gradient, with ordering one or more x-ray diffraction
30 holders and/or ordering one or more tools for excising and transporting a portion of the polymer matrix, such as one or more protein crystal transport tools for transporting a crystal-embedded polymer matrix section

to an x-ray diffraction holder, as disclosed herein.
For example, when a customer is directed to an Internet
page with a picture and/or description of a polymer
matrix with at least 2 regions of different pH, or when
5 a customer uses an on-line or phone-based means to
order a polymer matrix with at least 2 regions of
different pH, the customer is offered over the
telephone, or presented with an Internet link to
purchase one or more protein crystal transport tools
10 and/or one or more x-ray crystallography holders. In
another aspect, provided herein is a fee-based service
for performing the methods disclosed herein.

[0122] In yet a further aspect, the invention
provides kits for facilitating the practice of the
15 methods of the present invention.

[0123] In a first series of embodiments, the kit
comprises a polymer matrix having a continuous pH
gradient and instructions for performing the methods of
the present invention. In addition, the kit can
20 include a means for excising and transporting a portion
of the polymer matrix, such as one or more protein
crystal transport tools for transporting a crystal-
embedded gel piece to an x-ray diffraction holder. The
one or more protein crystal transport tools represent
25 another aspect of the invention. In certain aspects,
the polymer matrix includes a backing and is an IPG
strip.

[0124] The protein crystal transport tool(s) is
typically used to cut away or otherwise remove from the
30 remainder of the polymer matrix, a portion of the
polymer matrix that includes the protein crystals to a
size appropriate for an x-ray diffraction holder. The
protein crystal transport tool is then used to

transport the cut portion of polymer matrix that includes the protein crystals to an x-ray diffraction holder. The protein crystal transport tool can also be used, in certain embodiments, to separate the gel
5 matrix from a backing material. In certain aspects, several protein crystal transport tools are used to achieve these functions. For example, one tool can function as a cutter and another tool can function to pick up the cut portion and transfer it to the x-ray
10 diffraction holder. The tools may include a single handle or shaft that can be used interchangeably on a series of tips that perform the various functions.

[0125] The protein crystal transport tool has a polymer matrix contact portion and can also include a
15 handle or shaft in addition to the polymer matrix contact portion. As will be understood, the tool can take on any shape or size to achieve the functions stated above. For example, the protein crystal transport tool can take the general shape of a
20 miniaturized spade, knife, spatula, scraper, probe, chisel, shovel, scooper, or hoe, or can be a circular or cylindrical bore-type cutting tool that "punches out" a region of the polymer matrix that includes the protein crystals, similar in shape to micro tools known
25 in the art of protein crystal manipulation (See, for example, Micro Tools™ available from Hampton Research, Aliso Viejo, CA).

[0126] The protein crystal transport tool can be made of any material that is suitable for cutting and
30 transporting a polymer matrix without damaging protein crystals. For example, all or at least the polymer matrix contact region of the gel transport tool can be made of a materials that are known for crystal

transport, such as steel, for example stainless steel, or hardened tungsten steel. The handle or shaft, can be made of the same or a different materials than the polymer matrix contact region. For example, the handle
5 can be made of a material such as aluminum.

[0127] In certain aspects, the dimensions of the tools are greater than those used in protein crystal manipulation and smaller than tools used to excise gel portions for other purposes. The protein crystal
10 transport tool is typically capable of cutting the polymer matrix in a manner that is precise enough to remove a small section of a thin polymer matrix (e.g., a section that is about 1-5 mm in width, about 1-5 mm in length, and about 1-5 mm in thickness), and in
15 certain aspects is used to scrape a cut polymer matrix from a backing strip. In embodiments where the polymer matrix is attached to a backing, such as where IPG strips are used, the tool is typically rigid such that it slides along the stripping backing to lift the gel
20 from the backing. The tool typically includes one or more cutting edges that in certain illustrative embodiments are between 0.5 and 10 mm in length when the cutting edge is a straight edge. In aspects where the tool has a straight cutting edge, the tool can also
25 include a surface that is rectangular in shape. This surface is used to pick up and transport the gel piece, and therefore is typically flat or funneled toward a center portion. The area of this surface is typically between 5 mm² and 100 mm², and in certain illustrative
30 examples is between 10 mm² and 50 mm².

[0128] Where the tool is a bore-type cutting device, the cutting edge typically is capable of cutting a section of the gel that has an area of between 5 mm and

100 mm, or in certain illustrative examples between 10 mm and 50 mm. The bore-type cutting instrument can be used at the end of a capillary tube, which is used to collect and transport an excised portion of gel.

5 **[0129]** In embodiments, where the tool is a spatula-type tool, the front edge of the polymer matrix contact surface of the protein crystal transport tool can be the longest edge of the polymer matrix contact surface and should be no more than 100 mm wide. In
10 illustrative examples, the width of the front edge is 50, 25, 20, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, or 0.1 mm. For example, the width of the front edge of the polymer matrix contact surface of the protein crystal transport tool,
15 in certain illustrative examples, is between 0.1 mm and 10 mm. The other edges of the polymer matrix contact surface in spatula-type embodiments, typically have a width of 10,000, 5000, 2500, 1000, 500, 250, 100, 50, 25, 20, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.9, 0.8, 0.7,
20 0.6, 0.5, 0.4, 0.3, 0.2, or 0.1 mm. In certain aspects, the polymer matrix contact surface of a spatula-type tool has an area between 0.1 mm² and 10 cm², 0.5 mm² and 1 cm², or in an illustrative example, between 1 mm² and 100 mm².

25 **[0130]** The polymer matrix contact portion can include one surface, two surfaces that angle into contact with one another along a side edge or, for example, can include a bottom wall with two abutting side walls that are vertical with respect to the bottom
30 wall, can be cylindrical in shape, or can include various other configurations that can function as discussed herein. The portion of the tool that is used to transport the section of polymer matrix typically

includes at least one surface that is substantially horizontal, or horizontal, or includes a cylindrical cavity for holding a polymer matrix. Thus, the polymer matrix contact portion can be used to hold an excised portion of a polymer matrix on its top surface or within its body. The polymer matrix contact portion when part of a spatula-type tool, when used to transport a portion of a polymer matrix, holds the gel matrix in a position that is substantially parallel to the ground such that the polymer matrix does not fall from the protein crystal transport tool.

[0131] In certain embodiments, one or more x-ray diffraction holders are included in the kit. The x-ray diffraction holders can be virtually any type of x-ray diffraction holder known in the art. For example, the x-ray diffraction holder can be a cryo-loop.

Furthermore, the x-ray diffraction holder can be made of any suitable material, for example nylon. Accordingly, in certain embodiments, the kit includes a population of nylon fiber loops, such as cryo-loops. The number of x-ray diffraction holders included in the kit can be approximately equal (+/-2) or equal to the number of polymer matrices included in the kit. The x-ray diffraction holders in the kit are typically not reusable.

[0132] The kit can include an aqueous solvating composition, the solvating composition being adapted to and permissive for subsequent crystallization, according to the method of the present invention, of a protein species dissolved therein.

[0133] For example, the solvating composition will, in typical embodiments, lack detergent in amounts and of composition sufficient to denature a protein. The

solvating composition will typically lack chaotropic agents in amounts and of composition sufficient to denature a protein. The solvating composition will typically be of low ionic strength, and only slightly
5 buffered.

[0134] In some embodiments, the solvating composition will further comprise a non-detergent zwitterion, such as a non-detergent sulfobetaine, such as a sulfobetaine selected from the group consisting
10 of: dimethylethylammonium propane sulfonate; 3-(1-pyridino)-1-propane sulfonate; dimethylbenzylammonium propane sulfonate; 3-(4-*tert*-butyl-1-pyridino)-1-propane sulfonate; and 3-(1-methylpiperidinium)-1-propane sulfonate.

[0135] To facilitate the crystallization of proteins that may initially be present in compositions that are insufficiently permissive of crystallization according to the methods of the present invention, certain
15 embodiments of the kits of the present invention may include means for exchanging a protein into said
20 aqueous solvating composition.

[0136] The exchange means can, for example, be a centrifugation spin column with a semi-permeable membrane having a molecular weight cutoff below the
25 size of proteins desired to be crystallized. In some embodiments, the kit can include a plurality of exchange means, such as spin columns, each of the plurality having a semi-permeable membrane with a different molecular weight cutoff, so as to optimize
30 retention of the desired protein and passage of contaminants.

[0137] In a variety of embodiments, the polymer matrix is part of an IPG strip, and the kit comprises

one or more IPG strips. In typical embodiments, the kit comprises a selection of IPG strips having different continuous pH ranges, including both linear and nonlinear pH ranges, so as to facilitate the crystallization of a wide variety of proteins of disparate pI.

[0138] Usefully, the kit comprises means for effecting isoelectric focusing in IPG strips.

[0139] Thus, the kit may usefully further comprise means for enclosing at least one IPG strip, wherein the enclosing means permits spaced electrical communication separately with each of the at least one enclosed strips through respective first and second entries. In some embodiments, the enclosing means is capable of separately enclosing a plurality of IPG strips, permitting spaced electrical communication separately with each of the plurality of enclosed strips.

[0140] Conveniently, such kits can further comprise means responsive to an external compressive force for effecting spaced electrical communication by a single anode and single cathode simultaneously with each of the at least one enclosed strips, wherein the electrical communication means is capable of distributing an external compressive force to urge the anode and the cathode toward the enclosing means with greater pressure at the first and second entries than elsewhere on the enclosing means.

[0141] The kit may further comprise an electrophoresis tank, the tank dimensioned to accommodate both the means for enclosing IPG strips and the electrical communication means, colloquially denominated a buffer core.

[0142] The kits may also comprise means for excising a protein crystal from the polymer matrix, such as a coring tool.

[0143] The following examples are offered by way of illustration only, and not by way of limitation.

EXAMPLE 1

IPG Isoelectric Focusing Crystallization
of Soybean Trypsin Inhibitor

[0144] Soybean trypsin inhibitor was purchased from Sigma-Aldrich and dissolved in deionized water to a concentration of 10 mg/mL. An aliquot of 150 μ L of this solution was used to rehydrate a pH 4.5 to pH 5.5 ZOOM[®] Strip (Invitrogen Corp., Carlsbad, CA) in a ZOOM[®] IPGRunner cassette (Invitrogen Corp., Carlsbad, CA) for 2 hours.

[0145] The ZOOM[®] IPGRunner cassette was thereafter handled essentially according to manufacturer's instructions (including removal of well-forming members, application of wicks, and contact to an IPGRunner buffer core within an electrophoresis tank, all from Invitrogen Corp., Carlsbad, CA) and voltage applied as follows: 175V for 15 min, followed by a ramp from 175 to 2000 V over the course of one hour, and then 2000V for 2 hours 15 minutes.

[0146] Strips were removed from the cassette and examined microscopically. Crystals of soybean trypsin inhibitor were observed in the gel matrix, as shown in FIG. 1.

EXAMPLE 2

IPG Isoelectric Focusing Preparation of Seed Crystals

- 5 **[0147]** Lysozyme was purchased from Sigma-Aldrich and dissolved in deionized water at a concentration of 3 mg/mL. An aliquot of 150 microliters was used to rehydrate a pH 9 - 12 IPG strip (prepared in-house) within an Invitrogen ZOOM® IPGRunner cassette for 1 hour.
- 10 **[0148]** The ZOOM® IPGRunner cassette was thereafter handled essentially according to manufacturer's instructions (including removal of well-forming members, application of wicks, and contact to an IPGRunner buffer core within an electrophoresis tank,
- 15 all from Invitrogen Corp., Carlsbad, CA) and voltage applied using the following step voltage protocol: 175 volts for 15 minutes and 500 volts for 2 hours.
- 20 **[0149]** The strip was removed and examined microscopically. Micro-crystals of lysozyme were observed in the gel strip, as shown in FIG. 2A.
- 25 **[0150]** A section of the strip containing the micro-crystals was placed into a test tube containing a 22 mg/ml solution of lysozyme; at this concentration, lysozyme will not spontaneously crystallize within the tube.
- 30 **[0151]** After two days at room temperature, the strip was removed from the tube and examined microscopically: large crystals with similar morphology were observed as shown in FIG. 2B.

EXAMPLE 3

IPG IEF Crystallization After Buffer Exchange

[0152] Cytochrome P450 3A4 in a buffer containing 20 mM K_2PO_4 , 0.2 mM EDTA, 1 mM DTT, and 20% glycerol (PanVera Division of Invitrogen Corp.) was subjected to solution exchange into 0.3 mM CHAPS/0.3 mM octyl- β -D-1-thiogluco-pyranoside (OTGP) using a Centricon (YM-10) 10 kDa cutoff centrifuge tube from Millipore (Billerica, MA).

[0153] A pH 3 - 10 non-linear ZOOM[®] Strip (Invitrogen Corp., Carlsbad, CA) was rehydrated in the ZOOM[®] IPGRunner cassette for 1 hour with a volume of 150 μ L of 0.1 mg/mL of cytochrome P450 3A4 in the CHAPS/OTGP solution. Electrode wicks were wetted with 0.3 mM CHAPS/0.3 mM OTGP and applied to the cassette, essentially as per manufacturer's instructions.

[0154] The cassette was placed in the IPGRunner apparatus and run with an applied voltage of 200 volts for 1 hour. The strips were removed and examined microscopically. Crystals observed in the gel strip were mounted in thin-walled glass capillary tubes (0.7 mm in diameter) for x-ray crystallographic analysis. One such capillary-mounted crystal with which x-ray diffraction images were collected is shown in FIG. 3. The crystals appeared brown in color to the naked eye during and after isoelectric focusing without the use of a microscope, which provides evidence of the expected presence of native, non-denatured, concentrated, ligand-containing protein.

EXAMPLE 4

IPG IEF Crystallization under an Electrode

[0155] Hemoglobin S was purchased from Sigma-Aldrich and dissolved in dilute (0.125X) IEF Cathode Buffer

(Invitrogen Corp., Carlsbad, CA) to a concentration of 1 mg/mL. An aliquot of 150 μ L of this solution was used to rehydrate a pH 9-12 IPG strip (prepared in-house) within an Invitrogen ZOOM® IPGRunner cassette
5 (Invitrogen Corp., Carlsbad, CA) for 1 hour.

[0156] The IPGRunner cassette was thereafter handled essentially according to manufacturer's instructions (including removal of well-forming members, application of wicks, and contact to an IPGRunner buffer core
10 within an electrophoresis tank, all from Invitrogen Corp., Carlsbad, CA) with a constant voltage of 200 Volts applied for 4 hours to effect electrophoretic crystallization.

[0157] Following isoelectric focusing, electrode
15 wick fibers were disentangled from the gel matrix under the acidic electrode to reveal visible, gel-embedded crystals that were also examined microscopically as shown in FIG. 4.

[0158] A portion of the gel was first cut to
20 circumscribe one of the crystals, then scraped off the IPG strip backing, and finally scooped up by and onto a nylon fiber loop where the gel piece was dried at room temperature.

[0159] X-ray diffraction images were collected
25 directly using the loop-and-gel-mounted crystal. The crystals appeared red in color to the naked eye during and after isoelectric focusing without the use of a microscope, which provides evidence of the expected presence of native, non-denatured, concentrated,
30 ligand-containing protein.

[0160] All patents and publications cited in this specification are herein incorporated by reference as if each had specifically and individually been

incorporated by reference herein. Although the foregoing invention has been described in some detail by way of illustration and example, it will be readily apparent to those of ordinary skill in the art, in
5 light of the teachings herein, that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

What is Claimed is:

1. A method of crystallizing at least one protein, the method comprising:

electrophoretically focusing at least a first protein species within a matrix comprising at least 2 regions of different pH, the protein being present in amount sufficient to permit crystallization within said pH gradient.

2. The method of claim 1, wherein said focusing is performed in a matrix having an immobilized continuous pH gradient.

3. The method of claim 2, wherein said matrix is a polymer matrix.

4. The method of claim 3, wherein said polymer matrix comprises cross-linked acrylamide monomers.

5. The method of claim 4, wherein said continuous pH gradient is created by the covalent linkage within said polymer matrix of a plurality of species of buffering moieties having disparate pKa values.

6. The method of claim 5, wherein said plurality of species of covalently linked buffering moieties are a plurality of acrylamido monomer species having disparate pKa values.

7. The method of any one of claims 2 - 6, wherein said polymer matrix is adherent to at least one solid backing member.
8. The method of claim 7, wherein said polymer matrix is covalently attached to at least one of said at least one solid backing members.
9. The method of claim 8, wherein said polymer matrix is covalently attached to a single solid backing member.
10. The method of claim 7, wherein said at least one solid backing member is a flexible plastic.
11. The method of any one of claims 7 - 10, wherein said plastic backing member and the polymer matrix attached thereto is fashioned in the shape of strip that is greater in length along the axis of the pH gradient than in the dimensions orthogonal thereto.
12. The method of any one of claims 1 - 11, wherein said at least first protein species is initially present in inhomogeneous admixture with at least one additional protein species having a different isoelectric point.
13. The method of claim 12, wherein each of said protein species is concurrently crystallized within said matrix at its respective isoelectric focal point.

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14. The method of any one of claims 1 - 13, wherein said first and optional additional protein species are recombinantly expressed proteins.

15. The method of claim 14, wherein said recombinant expression is effected within a cell selected from the group consisting of: animal cell, fungal cell, insect cell, plant cell and prokaryotic cell.

16. The method of claim 14, wherein said recombinant expression is effected within a cell-free lysate.

17. The method of any one of claims 1 - 16, wherein said first and optional additional protein species are electrophoretically focused in the substantial absence of detergent.

18. The method of any one of claims 1 - 17, wherein said first and optional additional protein species are electrophoretically focused in the substantial absence of chaotropes.

19. The method of claim 17 or claim 18, wherein said first and optional additional protein species are electrophoretically focused in deionized water.

20. The method of claim 17 or claim 18, wherein said first and optional additional protein species are electrophoretically focused in the presence of a non-detergent zwitterion.

21. The method of claim 20, wherein said zwitterion is a sulfobetaine.

22. The method of claim 11 or of any one of claims 12 - 21 as dependent from claim 11, wherein said strip is a prior-cast, dehydrated, immobilized pH gradient (IPG) strip.

23. The method of claim 22, wherein electrophoretic focusing of said first and optional additional protein species comprises:

hydratingly lodging the prior-cast, dehydrated, IPG strip within an enclosing member that permits spaced electrical communication with said strip; and then

using said spaced electrical communication to establish a voltage gradient in the polymer matrix of said strip sufficient to effect electrophoretic focusing of proteins therein.

24. The method of claim 23, further comprising the prior step of inserting said prior-cast IPG strip in its dehydrated state within said enclosing member.

25. The method of claim 23 or claim 24, wherein said step of hydratingly lodging comprises:

contacting said enclosed dehydrated IPG strip with an aqueous solution for a time sufficient to lodge said separation medium within said enclosing member.

26. The method of claim 25, wherein said aqueous solution comprises said first and optional additional protein species to be crystallized.

27. The method of claim 25 or claim 26, wherein said lodging time is not more than about 2 hours.

28. The method of claim 27, wherein said lodging time is not more than about 1 hour.

29. The method of any one of claims 23 - 28, wherein said IPG strip isoelectric focusing is performed in a system comprising:

means for enclosing a plurality of said strips, said enclosing means permitting spaced electrical communication separately with each of said enclosed strips through respective first and second entries; and

means responsive to an external compressive force for effecting spaced electrical communication by a single anode and single cathode simultaneously with each of said enclosed strips, wherein said electrical communication means is capable of distributing an external compressive force to urge said anode and said cathode toward said enclosing means with greater pressure at said first and second entries than elsewhere on said enclosing means.

30. The method of any one of claims 1 - 29, wherein said first and optional additional protein

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species to be crystallized are focused for fewer than 5 hours.

31. The method of claim 30, wherein said first and optional additional protein species to be crystallized are focused for fewer than 3 hours.

32. The method of claim 31, wherein said first and optional additional protein species to be crystallized are focused for fewer than 2 hours.

33. The method of any one of claims 1 - 32, further comprising the antecedent step of:

partially purifying said first and optional additional protein species desired to be crystallized from an inhomogeneous protein mixture.

34. The method of claim 33, wherein said partial purification comprises fractionating by solution phase isoelectric focusing.

35. The method of any one of claims 1 - 34, further comprising the later step of:

isolating crystals of each of said first and optional additional protein species.

36. The method of claim 35, wherein said at least 2 regions of different pH are immobilized within said matrix, and isolating comprises separately excising the portion of said matrix in which said crystals of the first and optional additional crystallized protein species are embedded therein.

37. The method of any one of claims 1 - 36, further comprising the later step of:

washing said first and optional additional crystallized proteins with a solvent in which the protein remains crystallized.

38. The method of any one of claims 1 - 37, further comprising the step, after electrophoretically focusing, of:

using said crystallized first and optional additional protein species separately to nucleate further crystallization of each of said respective protein species from solution.

39. The method of any one of claims 1 - 38, further comprising a step of:

analyzing each of said first and optional additional crystallized protein species.

40. The method of claim 39, wherein said analysis comprises diffraction analysis.

41. The method of claim 40, wherein said diffraction analysis comprises X-ray diffraction analysis.

42. The method of claim 39, wherein said analysis comprises nuclear magnetic resonance analysis.

43. The method of any one of claims 39 - 42, wherein said analysis step comprises determining at least a partial 3D structure of said protein species.

44. The method of any one of claims 1 - 43, wherein said first and optional additional protein species is first complexed with a compound chosen from a chemical library.

45. A composition, comprising:
at least one crystal of at least a first protein species; and
a polymer matrix,
wherein the polymer matrix comprises a plurality of species of pH-conferring moieties, each of the plurality having a distinct pKa.

46. The composition of claim 45, wherein at least one of said at least one crystals of said at least first protein species is light microscopically observable.

47. The composition of claim 45 or claim 46, wherein at least one of said at least one crystals of said at least first protein species is of sufficient size to permit X-ray diffraction analysis.

48. The composition of any one of claims 45 - 47, wherein at least one of said at least one crystals of said at least first protein species is a crystal of the protein in a native, non-denatured, conformation.

49. The composition of claim 48, wherein said native, non-denatured, conformation is a conformation of the protein complexed with a non-protein ligand.

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50. The composition of claim 48, wherein said native, non-denatured, conformation is a conformation of the protein complexed with another protein species.

51. The composition of any one of claims 45 - 50, wherein said polymer matrix is an acrylamide polymer.

52. The composition of claim 51, wherein at least one of said plurality of pH-conferring moieties is an acrylamido buffer monomer.

53. The composition of claim 52, wherein each of said at least one acrylamido buffer monomers is selected from the group consisting of: 2-acrylamido-2-methylpropane sulfonic acid; 2-acrylamidoglycolic acid; N-acryloylglycine; 4-acrylamidobutyric acid; 2-morpholinoethylacrylamide; 3-morpholino-propylacrylamide; N,N-dimethylaminoethylacrylamide; N,N-dimethylaminoethylpropylacrylamide; and N,N-diethylaminopropylacrylamide.

54. The composition of any one of claims 45 - 53, wherein said polymer matrix is adherent to at least one support backing.

55. The composition of claim 54, wherein said polymer matrix is covalently attached to a single support backing.

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56. The composition of claim 55, wherein said support backing is a flexible plastic fashioned as a strip having greater length in the axis of the pH gradient than in directions orthogonal thereto.

57. The composition of any one of claims 45 - 56, wherein said plurality of species of pH-conferring moieties creates a continuous pH gradient along at least one dimension of said matrix.

58. The composition of claim 57, wherein said pH gradient has a pH range of no more than about 1 pH unit.

59. The composition of claim 57, wherein said pH gradient has a pH range of no less than about 7 pH units.

60. The composition of any one of claims 45 - 59, wherein said composition lacks detergent in an amount and of a composition sufficient to denature the first protein species.

61. The composition of any one of claims 45 - 60, wherein said composition lacks chaotrope in an amount and of a composition sufficient to denature the first protein species.

62. The composition of any one of claims 45 - 61, further comprising:
a non-detergent zwitterion.

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63. The composition of any one of claims 45 - 62, further comprising:

at least one crystal of a spatially segregated at least second protein species.

64. The composition of claim 63, wherein at least one of said at least one crystals of said at least second protein species is light microscopically observable.

65. The composition of claim 63 or claim 61, wherein at least one of said at least one crystals of said at least second protein species is of sufficient size to permit X-ray diffraction analysis.

66. A kit, comprising:

a polymer matrix having a continuous pH gradient; and at least one of:

instructions for performing the method of any one of claims 1 - 44, an x-ray diffraction holder, and a protein crystal transport tool.

67. The kit of claim 66, further comprising:

an aqueous solvating composition,

the solvating composition being permissive for subsequent crystallization according to the method of any one of claims 1 - 44 of a protein species dissolved therein.

68. The kit of claim 67, wherein said solvating composition lacks detergent in amounts and of composition sufficient to denature a protein.

69. The kit of claim 67 or claim 68, wherein said solvating composition lacks chaotropic agents in amounts and of composition sufficient to denature a protein.

70. The kit of any one of claims 67 - 69, wherein said solvating composition further comprises a non-detergent zwitterion.

71. The kit of claim 70, wherein said non-detergent zwitterion is a non-detergent sulfobetaine.

72. The kit of any one of claims 67 - 71, further comprising:
means for exchanging a protein into said aqueous solvating composition.

73. The kit of claim 72, wherein said exchanging means comprises a semi-permeable membrane.

74. The kit of any one of claims 66 - 73, wherein said polymer matrix is part of an IPG strip.

75. The kit of claim 74, further comprising:
means for enclosing at least one IPG strip,

wherein the enclosing means permits spaced electrical communication separately with each of the at least one enclosed strips through respective first and second entries.

76. The kit of claim 75, further comprising:

means responsive to an external compressive force for effecting spaced electrical communication by a single anode and single cathode simultaneously with each of the at least one enclosed strips, wherein the electrical communication means is capable of distributing an external compressive force to urge the anode and the cathode toward the enclosing means with greater pressure at the first and second entries than elsewhere on the enclosing means.

77. The kit of claim 76, further comprising:
an electrophoresis tank,
said tank dimensioned to accommodate said means for enclosing at least one IPG strip and said means responsive to an external compressive force.

78. The kit of any one of claims 66 - 77,
wherein the kit comprises a
protein crystal transport tool, whereby the tool is effective for excising a protein crystal from said polymer matrix and transporting the protein crystal to an x-ray diffraction holder.

79. The kit of claims 66, wherein the kit comprises an x-ray diffraction holder.

80. The kit of claims 74, wherein the kit comprises one or more protein crystal transport tools, which together are effective for excising a portion of the polymer matrix comprising the protein crystals and effective for transporting the portion of the polymer matrix comprising the protein crystals to an x-ray diffraction holder.

81. A method of crystallizing at least one protein, comprising electrophoretically transporting of at least a first protein species within a polymer matrix that includes at least 2 regions of different pH, wherein

the isoelectric point of the protein falls outside of the pH regions of the polymer matrix, and

the protein being present in an amount sufficient to permit formation of protein crystals at a terminal region of the polymer matrix.

82. The method of claim 81, wherein the polymer matrix comprises a continuous pH gradient

83. The method of claim 81, further comprising excising the terminal region of the polymer matrix that includes the protein crystals

84. The method of claim 83, further comprising analyzing the protein crystals using x-ray diffraction.

85. The method of claim 83, wherein the excision is performed using a protein crystal transport tool.

86. The method of claim 85, further comprising transporting of the excised terminal region of the polymer matrix to an x-ray diffraction holder.

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FIG. 1

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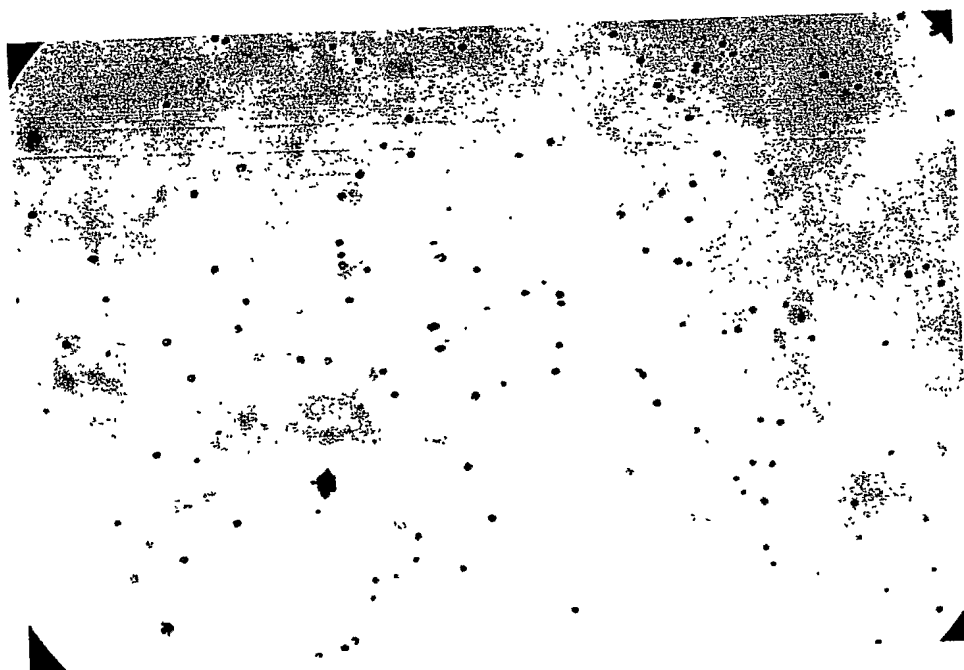


FIG. 2A

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FIG. 2B

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FIG. 3

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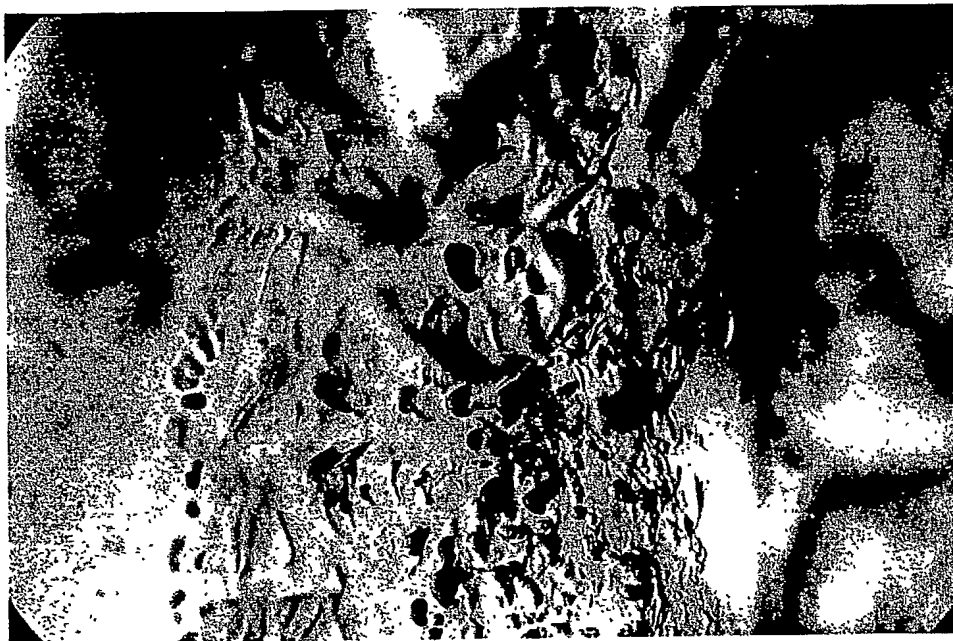


FIG. 4